

# Capillary Electrophoresis Analysis of Organic Amines and Amino Acids in Saline and Acidic Samples Using the Mars Organic Analyzer

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## Abstract

The Mars Organic Analyzer (MOA) has enabled the sensitive detection of amino acid and amine biomarkers in laboratory standards and in a variety of field sample tests. However, the MOA is challenged when samples are extremely acidic and saline or contain polyvalent cations. Here, we have optimized the MOA analysis, sample labeling, and sample dilution buffers to handle such challenging samples more robustly. Higher ionic strength buffer systems with  $pK_a$  values near pH 9 were developed to provide better buffering capacity and salt tolerance. The addition of ethylenediaminetetraacetic acid (EDTA) ameliorates the negative effects of multivalent cations. The optimized protocol utilizes a 75 mM borate buffer (pH 9.5) for Pacific Blue labeling of amines and amino acids. After labeling, 50 mM (final concentration) EDTA is added to samples containing divalent cations to ameliorate their effects. This optimized protocol was used to successfully analyze amino acids in a saturated brine sample from Saline Valley, California, and a subcritical water extract of a highly acidic sample from the Río Tinto, Spain. This work expands the analytical capabilities of the MOA and increases its sensitivity and robustness for samples from extraterrestrial environments that may exhibit pH and salt extremes as well as metal ions. **Key Words:** Amino acid analysis—Astrobiology—Planetary exploration—Lab-on-a-chip—Micro total analysis systems. *Astrobiology* 9, 823–831.

## Introduction

EXPLORATION OF MARS and other Solar System bodies for chemical signatures of life requires that we prepare for diverse and unexpected chemistries. In 1976, the Viking landers performed the first experiments to detect signs of past or present martian life. No organic molecules of martian origin were detected by its gas chromatograph–mass spectrometer (Biemann *et al.*, 1977), and the biology experiments were considered ambiguous (Levin and Straat, 1977; Oyama and Berdahl, 1977). These observations have been attributed to sterilizing radiation (Benner *et al.*, 2000), the presence of a strong oxidant in the regolith (Oyama and Berdahl, 1977; Zent and McKay, 1994), and the conversion of organic molecules to acids and amines during the pyrolysis extraction (Benner *et al.*, 2000; Glavin *et al.*, 2001). In 1997, the Pathfinder rover Sojourner characterized martian soil and rocks using X-ray fluorescence (Rieder *et al.*, 1997) and identified and quantified a range of minerals in the regolith, including MgO (8%), CaO (7%), and FeO (17%).

In 2004, the Mars Exploration Rover Opportunity discovered jarosite  $(K, Na)Fe_3(SO_4)_2(OH)_6$  (Klingelhöfer *et al.*, 2004) and other mineralogical evidence of ancient near-surface groundwater (Squyres *et al.*, 2004). The strong acidity and salinity of the ancient groundwaters suggested by Opportunity's data do not preclude the potential of extinct life. For example, terrestrial extremophilic organisms thrive in environments that range from the hypersaline (3–5 M NaCl) (Chaban *et al.*, 2006) to the highly acidic (pH < 2) (Cavicchioli, 2002; Chaban *et al.*, 2006) to the extremely cold ( $-17^\circ\text{C}$ ) (Cavicchioli, 2002). Furthermore, Phoenix recently observed extant martian water ice near the northern martian pole.

Evidence of saline and acidic environments has also been found on other astrobiological targets. The significant amounts of  $\text{CO}_2$  measured by Cassini in Enceladus' water-vapor plume (Waite *et al.*, 2006) may be indicative of subsurface chemistry rich in carbonic acid. Spectra taken by Galileo's near-infrared mapping spectrometer of Europa's surface suggest that Europa is paved in areas with hydrated

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salts (McCord *et al.*, 1998) and that sulfuric acid is a major component of its surface (Carlson *et al.*, 1999). These challenging environments suggest that organic molecules and potential biomarkers, if present, will be at very low concentrations; thus it will be critical for future planetary exploration instruments to be as sensitive as possible while remaining independent of the input sample chemistry.

In the search for extraterrestrial life, amino acids are well-justified target molecules due to their ubiquity in life on Earth and the large amount of information that can be obtained from measurement of their concentration, composition, and chirality (Lu and Freeland, 2006). Racemic mixtures of amino acids have also been found in a number of extraterrestrial sources, including martian meteorites (Becker *et al.*, 1999) and carbonaceous chondrite meteorites (Botta and Bada, 2002). Racemic mixtures indicate an abiological source, while homochiral amino acid composition provides strong evidence of extant or recently extinct life (Lu and Freeland, 2006); the degree of their racemization provides a measure of the age of extinct biota (Bada and McDonald, 1995). However, it is important not to limit our search to a single compound class. Amines (Skelley *et al.*, 2007), nucleobases (Skelley *et al.*, 2006), polycyclic aromatic hydrocarbons (Stockton *et al.*, 2009), and other organic molecules (Skelley and Mathies, 2003) are also important chemical markers and help to define whether the chemistry of an extraterrestrial sample is prebiotic, extant biotic, or extinct biotic.

The Mars Organic Analyzer (MOA) (Skelley *et al.*, 2005) is a portable lab-on-a-chip microcapillary electrophoresis ( $\mu$ CE) instrument that contains all the necessary microfluidics, optics, and electronics for capillary electrophoresis analysis. We have previously used the MOA to detect fluorescamine-labeled primary amines, amino acids (Skelley *et al.*, 2005), nucleobases (Skelley *et al.*, 2006), and polycyclic aromatic hydrocarbons (Stockton *et al.*, 2009), and we have used it to determine the chirality of amino acids (Skelley and Mathies, 2003). The MOA was successfully field tested in the Panoche Valley, California (Skelley *et al.*, 2005), and in the Atacama Desert (Skelley *et al.*, 2007). Recent work, in which Pacific Blue succinimidyl ester (PB) was used as the labeling reagent, has enhanced MOA sensitivity to the parts-per-trillion level (Chiesl *et al.*, 2009). PB labeling also enables the performance of micellar electrokinetic chromatography (MEKC) for pseudo-2-dimensional separations and thus dramatically enhances compositional analysis (Chiesl *et al.*, 2009).

The application of the MOA to real-world samples requires that we enhance the robustness of the assay to varying sample characteristics. For example, samples with extreme salinity cause dispersive effects during  $\mu$ CE injection and lead to poor resolution and signal loss (Landers, 1997). Extremely acidic samples provide challenges due to inhibition of the labeling reaction (De Bernardo *et al.*, 1974) and cessation of the electro-osmotic flow in the separation channels, both of which dramatically reduce signal. Furthermore, multivalent cations can alter the surface chemistry of the  $\mu$ CE channels and also lead to dispersive effects and electro-osmotic flow inhibition (Brechtel *et al.*, 1995). Since the concentrations of organic biomarkers expected on Mars may be low, it is crucial to enhance the MOA's tolerance to highly saline, acidic, or ionic samples. Here, we explore the assay conditions and buffers that are necessary to analyze samples containing high levels of acid, salts, and multivalent cations,

and determine optimal labeling and analysis protocols. These optimized formats are then used to analyze two samples from representative extreme environments: saturated brine from Saline Valley, California, and a subcritical water extract from the acidic Río Tinto in Spain.

## Materials and Methods

### Buffer and sample preparation

Lithium carbonate, sodium tetraborate, and sodium phosphate were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of 100 mM carbonate, 100 mM phosphate, and 400 mM borate were prepared. The carbonate stock was adjusted to pH 8.5 with 100 mM HCl. Phosphate (pH 9.0) and borate (pH 9.5) were used without pH adjustment. Both the disodium and tetrasodium salts of ethylenediaminetetraacetic acid (EDTA) were obtained from Fischer Scientific (Pittsburgh, PA) and combined to produce a 250 mM, pH 7.0 aqueous stock solution. Racemic stock solutions (20 mM in water) were prepared for each amino acid (Sigma-Aldrich) and combined in appropriate volumes. Pacific Blue succinimidyl ester (ex. 405 nm, em. 425 nm) was obtained from Invitrogen (Carlsbad, CA), dissolved to 20 mM in *N,N*-dimethylformamide, and stored at  $-20^{\circ}\text{C}$ . Fluorescamine (Sigma-Aldrich) stock solution (20 mM) was prepared in dimethylsulfoxide (Sigma-Aldrich).

A stock solution of an amino acid standard containing equal 40  $\mu\text{M}$  concentrations of citrulline, valine, serine, alanine, glycine, aspartic acid, and glutamic acid was prepared. Labeling reactions were conducted by combining one part each of the amino acid stock and buffer, then adding two parts PB stock solution. The labeling mixture was allowed to react overnight at room temperature before dilution for analysis. This standard was diluted to 400 nM of each amino acid with the appropriate buffers for each experiment. Analysis was conducted by filling the separation column and wells with the analysis buffer then replacing the solution in the sample well with the diluted, labeled amino acid standard. Electropherograms were analyzed with PeakFit to determine the signal-to-noise, resolution, and peak efficiency in theoretical plates per meter for each amino acid peak.

To determine the effect of EDTA on labeling reaction, buffer solutions were prepared containing EDTA concentrations that ranged from 0 to 200 mM in 30 mM borate buffer (pH 9.5). Reactions containing 20  $\mu\text{M}$  glycine and 400  $\mu\text{M}$  fluorescamine were conducted in these buffer solutions. After a 5-minute reaction, each sample was diluted to a final concentration of 133 nM glycine, 2.7  $\mu\text{M}$  fluorescamine in its respective buffer. Fluorescence intensity measurements were then taken on a Jasco FP-750 spectrofluorometer.

Saline Valley and Río Tinto samples were labeled by a standard protocol and an optimized protocol. The optimized protocol consisted of combining 5  $\mu\text{l}$  of the sample with 20  $\mu\text{l}$  75 mM borate (pH 9.5) and adding 10  $\mu\text{l}$  PB stock. After an overnight reaction at room temperature, water and 250 mM EDTA were added to the labeled Río Tinto sample to provide a final 10-fold dilution with final EDTA and borate concentrations of 50 mM and 30 mM, respectively. The Saline Valley sample was allowed to react for 30 min; water was then added to provide a final 10-fold dilution of the sample with borate concentration of 30 mM. The standard method was conducted in an identical manner, with use of 4 mM car-

bonate buffer (pH 8.5) in place of all borate and borate/EDTA buffers. Samples labeled via the optimized method were analyzed with 30 mM borate in the separation column, and samples labeled via the standard method were analyzed with 4 mM carbonate in the separation column.

Salt sample SV07-4 was obtained from Saline Valley, California (Lowenstein, 2007; Schubert *et al.*, 2009), and was labeled according to both the standard method and the optimized protocol. A sediment sample from the Río Tinto, KF03-A, collected from a similar region as KF03-136 (Fernández-Remolar *et al.*, 2005), was obtained as part of the Astrobiology Sample Analysis Program (ASAP) (Glavin *et al.*, 2008). A subcritical water extraction was performed by collaborators at the Jet Propulsion Laboratory on 500 mg of sample (Amashukeli *et al.*, 2007). One-sixteenth of the total extract was freeze-dried and resuspended in 400  $\mu$ l 4 mM carbonate, pH 8.5 for analysis.

#### Microdevice fabrication

The microdevices were prepared as previously described (Skelley and Mathies, 2003; Skelley *et al.*, 2005, 2006; Stockton *et al.*, 2009). In summary, a sacrificial layer of polysilicon was deposited via chemical vapor deposition on a 10 cm borofloat glass wafer. A layer of photoresist was then spin-coated onto the wafer and patterned through a chrome mask by using a contact aligner. After the photoresist was developed, the exposed polysilicon was removed by plasma etching. Etching in buffered HF produced 23.6 cm long folded separation channels 100  $\mu$ m wide and 25  $\mu$ m deep. After removing photoresist, reservoir holes were drilled, the polysilicon layer was removed, and the wafer was bonded to a blank wafer to form completed channels. Injections were performed via a 1.2 cm long cross channel located 0.6 cm from the anode end of the channel. A 3 mm deep polydimethylsiloxane gasket with 4 mm diameter wells was placed over the reservoir holes to allow for larger volume. A schematic of the microdevice has been published and is presented in Supplemental Data (Chiesl *et al.*, 2009; Stockton *et al.*, 2009).

#### Mars Organic Analyzer

The Mars Organic Analyzer (Skelley *et al.*, 2005) was used with minor modifications to the optical subsystem that allow for more sensitive analysis of amino acids (Chiesl *et al.*, 2009; Stockton *et al.*, 2009). The 404 nm laser was passed through a dichroic and focused to a 10–20  $\mu$ m spot in the channel approximately 0.6 cm from the cathode reservoir. Fluorescence was collected by the objective and reflected by the dichroic through a long-pass filter onto a photomultiplier tube, which was digitized at 50 Hz.

#### Separation and injection procedures

The microchip separation channel was prepared by first filling the sample, waste, and anode wells with running buffer. A vacuum was applied to the cathode well, which drew running buffer into the separation channel. The cathode well was then filled with running buffer, and the buffer in the sample well was drawn out and replaced with sample. Cross injection was accomplished by first applying a potential across the sample (ground) and waste (–1000 V) wells for 30 s with the anode and cathode grounded. This was followed by a

200 ms step where the anode was floated. The separation was performed by applying –15 kV at the cathode, –1.4 kV at the sample and waste, and ground at the anode.

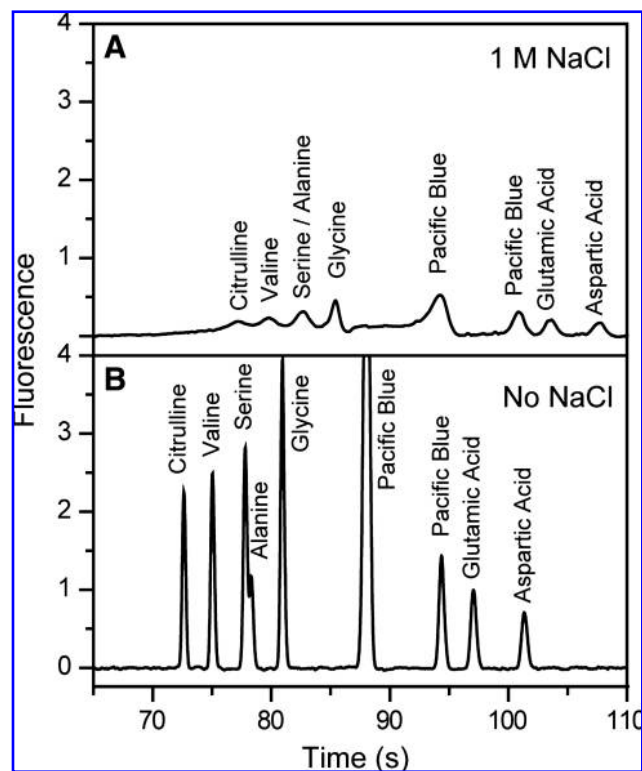
## Results

#### Initial buffer system selection

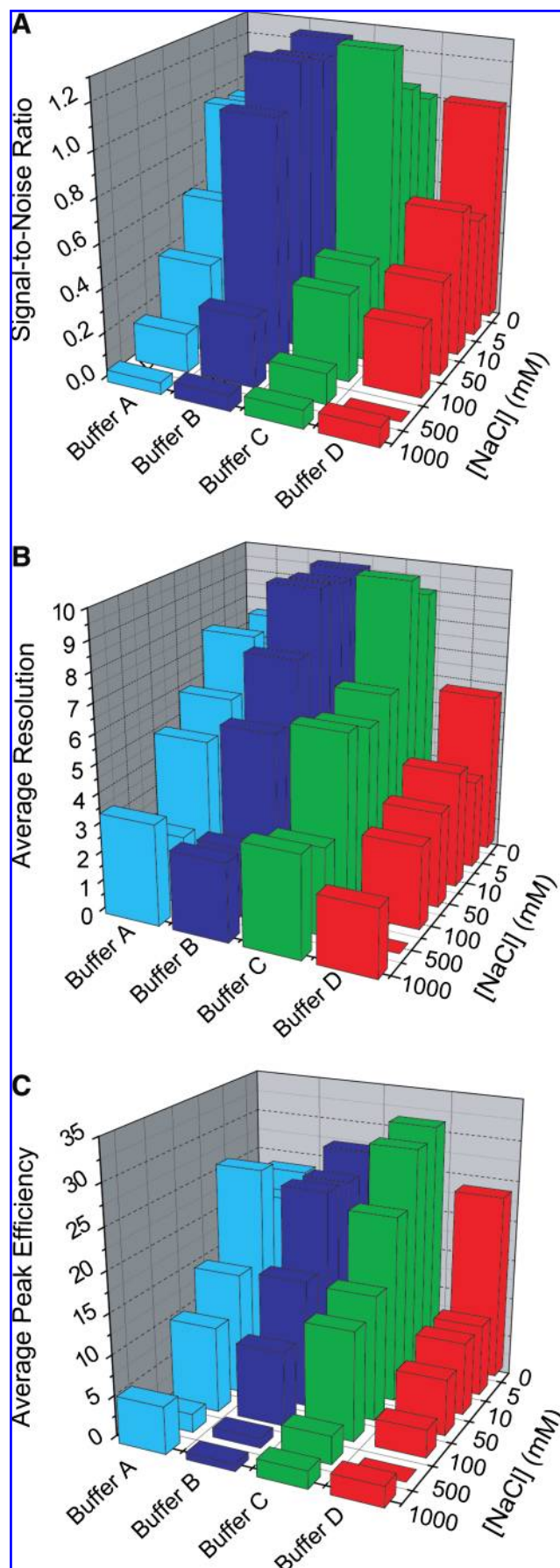
The fluorescamine labeling reaction has an optimal pH range between 8.0 and 10.0 (De Bernardo *et al.*, 1974). Since PB labeling proceeds via a similar reaction mechanism, the reaction buffers chosen for study had  $pK_a$  values near the same range. Phosphoric acid has a  $pK_a$  at 7.21 associated with the equilibrium between its diprotonated anion and monoprotonated anion. Borate has a  $pK_a$  value of 9.23 due to its interaction with water. Each of these options provides a more suitable choice than carbonate ( $pK_a$  values 6.37, 10.25). Unacceptably high separation currents were observed with buffers containing greater than 50 mM borate or 10 mM phosphate concentrations. The following three buffer systems were chosen for study: 30 mM borate in both the sample and the separation channel, 30 mM borate in the separation channel with 5 mM borate in the sample, and 5 mM phosphate in the separation channel with 30 mM borate in the sample.

#### Effects of NaCl in the sample

Electropherograms shown in Fig. 1 reveal the effect of high concentrations of salt in the sample. The high-salt



**FIG. 1.** Electropherograms of an amino acid standard with (A) and without (B) 1 M NaCl. The separation channel and sample buffer contained 5 mM borate buffer, pH 9.5. The standard contained 2  $\mu$ M each citrulline, valine, serine, alanine, glycine, aspartic acid, and glutamic acid labeled with Pacific Blue.



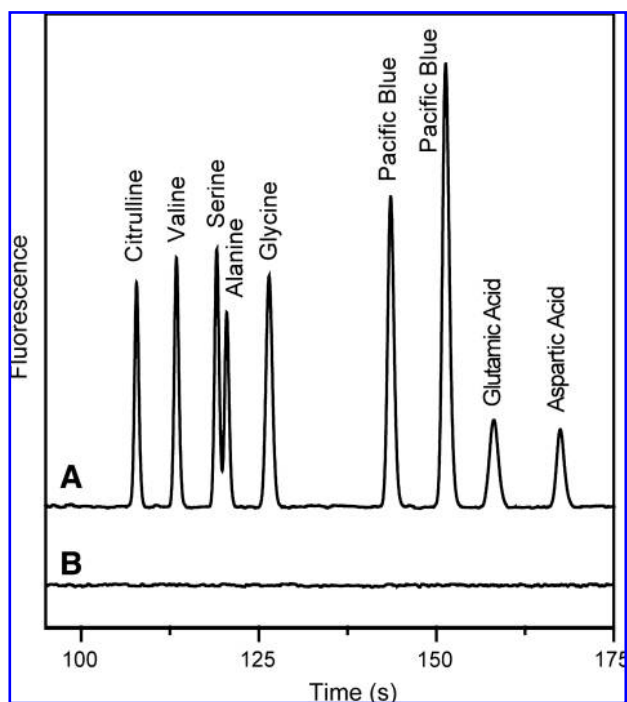
sample exhibits lower signal, broader peaks, and reduced resolution compared to the experiment performed with no NaCl in the sample solution. The signal-to-noise ratios, resolutions, and peak efficiencies for the amino acids were then determined for each NaCl concentration and buffer system and are summarized in Fig. 2. All four buffer systems showed a significant decline in all three parameters with increasing sample NaCl, with the 5 mM borate system (Buffer D) displaying the most significant decline. While the buffer system containing 5 mM phosphate in the separation channel and 30 mM borate in the sample (Buffer B) retained the best signal-to-noise ratio with increasing sample NaCl, the buffer system containing 30 mM borate in both the separation channel and the sample (Buffer C) retained the best resolution and peak efficiencies. Because of its operational simplicity, the latter buffer system was chosen for further optimization.

#### Effects of multivalent cations and EDTA

Studies of samples containing additional contaminating salts, including  $\text{Na}_2\text{SO}_4$ ,  $\text{MgCl}_2$ ,  $\text{NaClO}_4$ ,  $\text{FeCl}_3$ , and  $\text{CaCl}_2$ , indicated that the anion has a negligible effect on the separation quality. However, multivalent cations were found to reduce the signal significantly even at concentrations as low as 5 mM. We therefore added EDTA to the sample buffer to chelate these cations and counteract their effect, as illustrated with the use of  $\text{Mg}^{2+}$  as a model cation in Fig. 3. The sample used for the top electropherogram (A) contained both 5 mM  $\text{MgCl}_2$  and 10 mM EDTA; full signal strength and high separation quality were observed. The 5 mM  $\text{MgCl}_2$  sample used for the bottom trace (B) did not contain EDTA; therefore, signal was not obtained. An optimization experiment was conducted varying the EDTA and  $\text{MgCl}_2$  concentration (Fig. 4). When the  $\text{MgCl}_2$  concentration was higher than the EDTA concentration, no signal was observed, as expected. When the  $\text{MgCl}_2$  concentration was equal to the EDTA concentration, some peaks were observed, but with lower signals and reduced resolution and peak efficiency. When the EDTA concentration was greater than the  $\text{MgCl}_2$  concentration, signal, peak efficiency, and resolution were restored. However, the signal and other performance characteristics tended to decrease as the EDTA concentration was further increased. Experiments in which  $\text{FeCl}_3$  (Supplemental Data Fig. S03) was used exhibited similar trends.

**FIG. 2.** Effects of sample salt content on separation performance with use of selected buffering systems. Buffer A consisted of 30 mM borate in the separation channel and 5 mM borate in the sample. Buffer B consisted of 5 mM phosphate in the separation channel and 30 mM borate in the sample. Buffer C consisted of 30 mM borate in both the separation channel and the sample. Buffer D consisted of 5 mM borate in both the separation channel and the sample. (A) Average signal-to-noise of all amino acid peaks normalized to the no-salt case for each buffer. (B) Average resolution between amino acid peaks for each buffer system at the indicated concentrations of NaCl. (C) Average amino acid peak efficiency in theoretical plates-per-meter ( $\times 10^{-4}$ ) for each buffer system at the indicated concentrations of NaCl. The sample is the same as Fig. 1. Color images available online at [www.liebertonline.com/ast](http://www.liebertonline.com/ast).



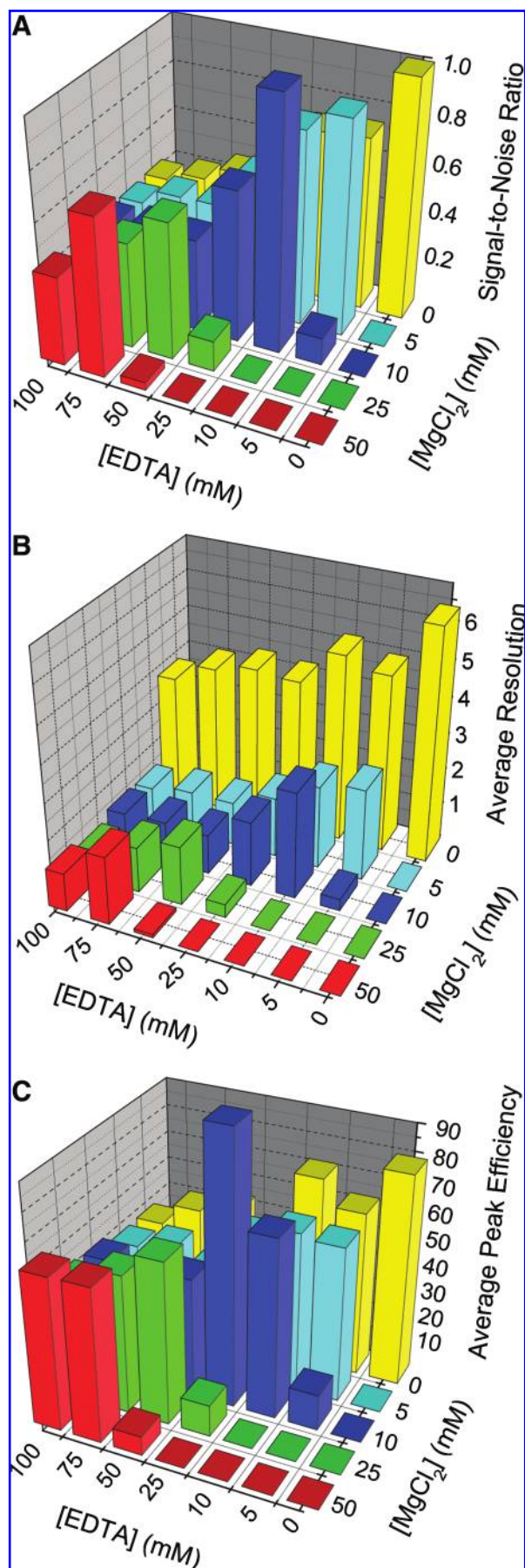


**FIG. 3.** Electropherograms of a standard containing 5 mM  $\text{MgCl}_2$  with 10 mM EDTA (A) and without EDTA (B). The separation column and sample contained 30 mM borate buffer, pH 9.5. The sample contained 2  $\mu\text{M}$  each citrulline, valine, serine, alanine, glycine, aspartic acid, and glutamic acid labeled with Pacific Blue.

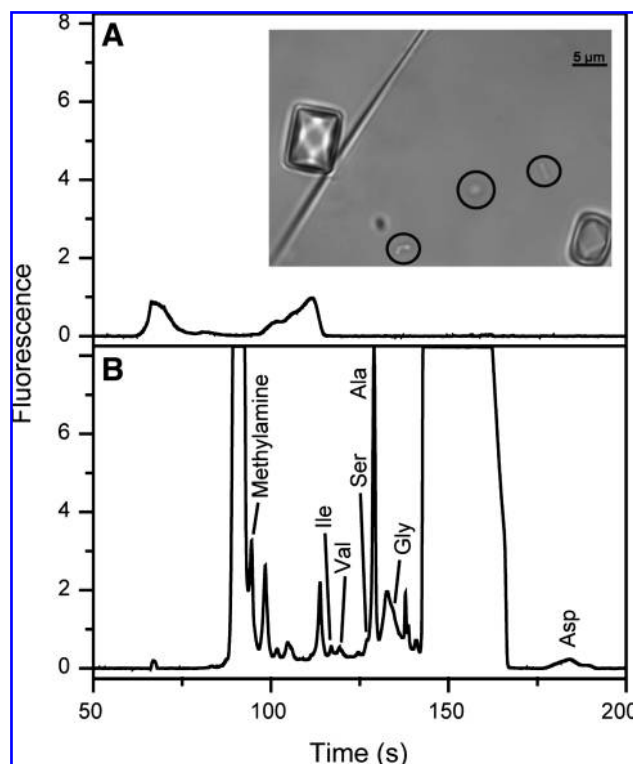
Based on these results, an EDTA concentration of 50 mM was chosen as the optimal concentration for further studies.

#### Effects of EDTA on labeling

The fluorescent signal of a solution of an amino acid and fluorescamine provides a convenient measure of the extent of the amino acid labeling reaction. Fluorescence measurements were made in buffers containing 30 mM borate and EDTA concentrations that ranged from 0 to 200 mM (Fig. S04 in Supplemental Data). There was a significant decline in labeling efficiency in samples containing EDTA; the higher the concentration of EDTA, the stronger the inhibition. While exploring the reason for EDTA inhibition of the labeling reaction is beyond the scope of this work, it may be due to decreased cationic species available to stabilize the negatively charged tetrahedral intermediate formed when an amino compound attacks the carbonyl carbon in the first step of the labeling reaction mechanism. We conclude that the



**FIG. 4.** Effects of different EDTA and  $\text{MgCl}_2$  concentrations in the sample buffer on separation performance. The separation channel and sample contained 30 mM borate buffer, pH 9.5. (A) Average signal-to-noise of all amino acid peaks normalized to the no-salt, no-EDTA case. (B) Average resolution between amino acid peaks for each concentration of EDTA and  $\text{MgCl}_2$ . (C) Average peak efficiency in theoretical plates-per-meter ( $\times 10^{-4}$ ) of amino acid peaks for each concentration of EDTA and  $\text{MgCl}_2$ . The sample is the same as in Fig. 3. Color images available online at [www.liebertonline.com/ast](http://www.liebertonline.com/ast).



**FIG. 5.** Analysis of Saline Valley brine SV07-4. **(A)** Electropherogram of Pacific Blue labeled sample with the separation channel and sample containing 4 mM carbonate, pH 8.5. The inset presents a photomicrograph of a wet mount viewed with a 100 $\times$  oil immersion lens. Prokaryote cells occur as rod and coccoid shapes (circled); halite (cube) and mirabilite (needle) crystallized from the Na-Cl-SO<sub>4</sub>-rich brine after collection. Scale bar is 5  $\mu$ M. **(B)** Electropherogram of Pacific Blue labeled sample, where the separation channel contained 30 mM borate, pH 9.5, and the sample contained 30 mM borate, 50 mM EDTA, pH 9.5. Ala, alanine; Asp, aspartic acid; Gly, glycine; Ile, isoleucine; Ser, serine; Val, valine.

optimal labeling buffer should not contain EDTA because the positive effects of EDTA can be achieved by dilution after reaction.

#### Analysis of Saline Valley and Río Tinto samples

Two samples that exhibited extreme salinity and acidity were chosen to test the robustness of the optimized buffer system. The high-salinity sample was a halite saturated brine, SV07-4, from Saline Valley, California (Fig. 5). This sample was of particular interest because it contained halophilic archaea and bacteria (Schubert *et al.*, 2009). An image of the brine taken with a 100 $\times$  oil-immersion lens shows several prokaryote cells (rod and coccoid shapes) accompanying cubic halite (NaCl) crystals and a mirabilite (Na<sub>2</sub>SO<sub>4</sub>10H<sub>2</sub>O) needle (Fig. 5A). Despite the abundance of prokaryote cells, electropherograms of the MOA analysis of these Na-Cl-SO<sub>4</sub>-rich brines via the standard protocol (Fig. 5A) exhibit extremely low signal amino acid peaks because of the high salt concentration (saturated with halite and mirabilite). Electropherograms obtained via the optimized protocol show significantly increased peak intensities (Fig. 5B), which allow for the identification and quantification of

**TABLE 1.** AMINO ACID ANALYSIS OF CHALLENGING SAMPLES

Amine/amino acid	Río Tinto <sup>a</sup> (KF03-136, ppb)	Saline Valley <sup>b</sup> (SV07-4, $\mu$ M)
Ammonia	9,520	ND <sup>c</sup>
Methylamine	809	200
Ethylamine	3,640	ND <sup>c</sup>
Leucine + Isoleucine	136	ND <sup>c</sup>
Valine	418	4.3
Serine	19,800	11
Alanine	855	210
Glycine	6,200	0.48
Aspartic acid	717	27

<sup>a</sup>Composition calculated as a fraction of initial sample assuming 100% recovery during the subcritical water extraction and subsequent processing.

<sup>b</sup>Concentrations of detected species in the received saturated brine solution.

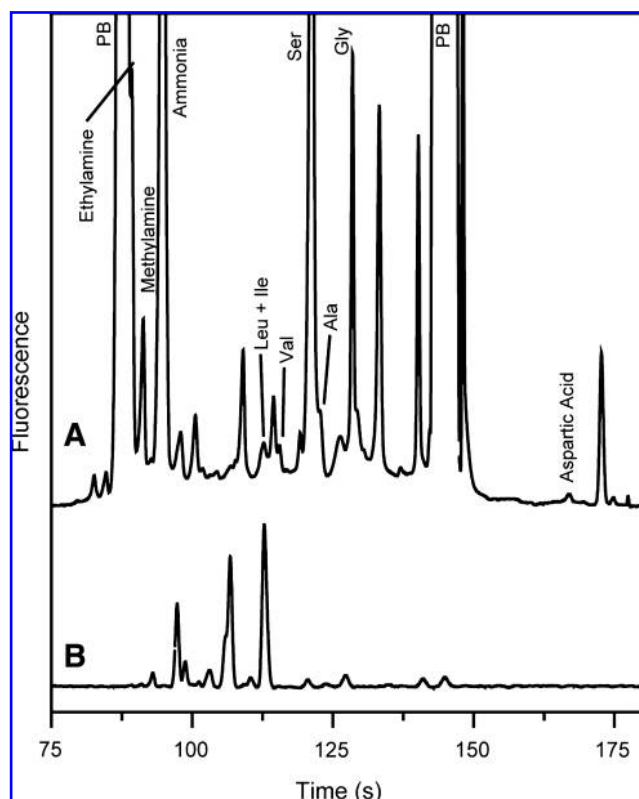
<sup>c</sup>Species not reported because either they were not observed or they were observed with signal at or below that in a buffer blank.

multiple amino acids in the brine solution. The amino acid composition and concentrations in this sample are shown in Table 1.

The Río Tinto in Spain provides an example of both high salinity and extreme acidity. The sample pH is less than 2, and it contains high iron, magnesium, and general salts with concentrations greater than 500 mM (Fernández-Remolar *et al.*, 2005), which makes  $\mu$ CE amino acid analysis challenging. A subcritical water extract of the Río Tinto sample KF03-A was analyzed with the standard and optimized assays. Electropherograms obtained with the standard protocol (Fig. 6B) exhibit low signal and an absence of peaks eluting after  $\sim$ 150 s. However, analysis in which the optimized buffer system was used (Fig. 6A) showed much stronger amine and amino acid signals, allowing for substrate identification and quantification (Table 1). In KF03-A, ammonia, ethylamine, serine, and glycine were present in parts-per-trillion levels, while methylamine, valine, alanine, and aspartic acid were present in high parts per billion. The complexity of the trace and the large levels of amines and amino acids in this sample were likely derived from a thriving acidophile population. This improvement in analytical capability was due to improved buffering against the high acid concentration, chelation by EDTA of multivalent cations (*e.g.*, Mg<sup>2+</sup>, Fe<sup>3+</sup>), and increased resistance to deleterious salt effects in the injection and separation.

#### Discussion

The optimized MOA analysis protocol developed here provides several benefits over our previous methods. The 5 mM borate buffer system used in the salt experiments is most similar to the previously used 4 mM carbonate buffering system (Chiesl *et al.*, 2009). With no NaCl present in the sample, there was no mismatch in ionic strength between the sample matrix and the separation buffer, so there was little difference between the 5 mM borate buffer and the other buffer systems tested. However, at higher sample NaCl concentrations, the mismatch in ionic strengths of the sample and separation column produced intense dispersive effects when using the 5 mM borate system. Because the ionic



**FIG. 6.** Electropherograms of a Pacific Blue labeled subcritical water extract of the Río Tinto sample KF03-A (Fernández-Remolar *et al.*, 2005). The sample was diluted 5-fold with sample buffer before labeling then analyzed at a total dilution factor of 10-fold. (A) Separation channel contained 30 mM borate, pH 9.5. The sample was diluted with 30 mM borate before labeling then brought to a final sample buffer composition of 30 mM borate, 50 mM EDTA for injection. (B) Separation and sample buffer are both 4 mM carbonate, pH 8.5. Ala, alanine; Gly, glycine; Ile, isoleucine; Leu, leucine; Ser, serine; Val, valine.

strength of the separation buffer was higher for the 30 mM borate system, the mismatch was reduced, and dispersive band broadening was less pronounced.

The 30 mM borate system has a larger capacity than the 4 mM carbonate system, so it can buffer a significantly larger concentration of acid or base present in a sample. The larger buffer capacity is partly due to the higher concentration of buffer salt: 30 mM borate in the optimized system compared to 4 mM carbonate in the previous system. However, it also arises from a better choice of the buffer  $pK_a$  values relative to the operating pH. The carbonate buffer was used at pH 8.5, which is well out of its effective buffering range ( $pK_a$  6.4). The borate buffer has a  $pK_a$  (9.23) that is significantly closer to the operating pH of 9.5.

To ensure that analysis is not compromised by the presence of divalent cations, it is essential to have some EDTA in the sample buffer. While the presence of EDTA reduces signal, the complete loss of signal seen with even a 5 mM addition of  $Mg^{2+}$  makes the 10–50% EDTA-induced loss in signal and separation quality acceptable. It is also interesting to note that divalent cations appear to counter the deleterious effect of EDTA on the separation, as nearly full signal,

resolution, and peak efficiency are restored when the EDTA concentration is just higher than the  $MgCl_2$  concentration. Although EDTA also has an inhibitory effect on sample labeling, it is easy to circumvent this problem by adding EDTA to the sample after the labeling reaction is complete.

The net benefits of the optimized method over the standard method are best demonstrated by the analysis of terrestrial samples with extremes of salinity and pH. The high-salt sample used was brine taken from the saline pan of Saline Valley. The sampling site hosts a diverse community of single-celled halophilic algae (genus *Dunaliella*) and halophilic bacteria and archaea; thus we would expect large concentrations of amino acids (Lowenstein, 2007; Schubert *et al.*, 2009). This sample was pH neutral and did not contain significant levels of divalent cations, so high salt concentrations ( $Na^+$ ,  $Cl^-$ , and  $SO_4^{2-}$  at halite and mirabilite saturation) were the major concern. Analysis with our previous method showed low signal and peak broadening, which makes amino acid identification and quantitation impossible. The analysis via the optimized protocol, however, allowed for identification of several amino acids and quantitation of total amino acid content at 450  $\mu M$ . This result is a significant advance in the MOA capabilities because it demonstrates the potential for the direct analysis of aqueous extracts without extensive preprocessing (Kminek *et al.*, 2000; Amashukeli *et al.*, 2007).

The Río Tinto in Spain has a pH less than 2 and contains high concentrations of metal salts (Amaral Zettler *et al.*, 2002). Despite these seemingly hostile characteristics, the Río Tinto hosts a rich ecosystem, with a surprising degree of eukaryotic diversity (Amaral Zettler *et al.*, 2002), and thus high levels of amino acids and organic amines are expected (Glavin *et al.*, 2008). The extreme acidity of this concentrated ASAP (Glavin *et al.*, 2008) sample severely inhibited labeling via the previous protocol, and the high metal content severely limited the injection, which resulted in low signal. In our Río Tinto extract analysis for the ASAP (Glavin *et al.*, 2008), the sample was manually neutralized before labeling and analyzed via the standard protocol, but the electropherogram still shows low signal strength because of the high salt concentration resulting from neutralization and the high metal content.

In contrast, the optimized method employed here to analyze the Río Tinto sample required no neutralization before labeling and produced traces with high signal strength. This improvement is seen because of the much larger buffering capacity of the optimized buffering system and the addition of EDTA to the sample buffer after labeling to negate effects of the sample's exceptionally high metal content. The unusually high abundance of serine in this sample raises the question whether the subcritical water extraction method, salts, a coeluting amine, or an analytical artifact may increase the intensity of this peak. While this assignment was confirmed by coelution of a serine spike, additional 2-dimensional capillary electrophoresis separations with MEKC (Chiesl *et al.*, 2009) and mass spectrometry would provide valuable confirmation.

The optimized buffer system developed here should also be useful for chiral separations of labeled amino acids. The improved buffering system should facilitate separation of fluorescamine labeled amino acids in cyclodextrin buffers that have been shown to provide chiral resolution (Skelley



and Mathies, 2003). Chiral separations of PB labeled amino acids conducted with MEKC (Chiesl *et al.*, 2009) should also exhibit decreased sensitivity to sample salt and pH once the detailed conditions are optimized.

## Conclusions

This work demonstrates that proper buffering is critical for successful sample analysis and that amino acid extracts obtained from samples from extreme environments can be directly injected into the MOA  $\mu$ CE instrument and analyzed. These new methods critically advance the development of our analytical system for biomarker detection providing broad applicability and versatility for planetary exploration. For more information on MOA development, see <http://astrobiology.berkeley.edu>.

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## Author Disclosure Statement

A.M. Stockton, T.N. Chiesl, T.K. Lowenstein, X. Amashukeli, F. Grunthaner, and R.A. Mathies declare no competing financial interest.

## Abbreviations

ASAP, Astrobiology Sample Analysis Program; EDTA, ethylenediaminetetraacetic acid;  $\mu$ CE, microcapillary electrophoresis; MEKC, micellar electrokinetic chromatography; MOA, Mars Organic Analyzer; PB, Pacific Blue succinimidyl ester.

## Supplemental Data

Additional data as noted in the text is available online at [www.liebertonline.com/ast](http://www.liebertonline.com/ast).

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